Insulin-like growth factor-binding protein-3 inhibition of prostate cancer growth involves suppression of angiogenesis

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Insulin-like growth factor-binding protein-3 (IGFBP-3) is a multifunctional protein that induces apoptosis utilizing both insulin-like growth factor receptor (IGF)-dependent and -independent mechanisms. We investigated the effects of IGFBP-3 on tumor growth and angiogenesis utilizing a human CaP xenograft model in severe-combined immunodeficiency mice. A 16-day course of IGFBP-3 injections reduced tumor size and increased apoptosis and also led to a reduction in the number of vessels stained with CD31. *In vitro*, IGFBP-3 inhibited both vascular endothelial growth factor- and IGF-stimulated human umbilical vein endothelial cells vascular network formation in a matrigel assay. This action is primarily IGF independent as shown by studies utilizing the non-IGFBP-binding IGF-1 analog Long-R3. Additionally, we used a fibroblast growth factor-enriched matrigel-plug assay and chick allantoic membrane assays to show that IGFBP-3 has potent antiangiogenic actions *in vivo*. Finally, overexpression of IGFBP-3 or the non-IGF-binding GGG-IGFBP-3 mutant in Zebrafish embryos confirmed that both IGFBP-3 and the non-IGF-binding mutant inhibited vessel formation *in vivo*, indicating that the antiangiogenic effect of IGFBP-3 is an IGF-independent phenomenon. Together, these studies provide the first evidence that IGFBP-3 has direct, IGF-independent inhibitory effects on angiogenesis providing an additional mechanism by which it exerts its tumor suppressive effects and further supporting its development for clinical use in the therapy of patients with prostate cancer.

Keywords: insulin-like growth factor-binding protein-3; prostate cancer; angiogenesis; apoptosis

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Introduction

Regulation of cellular growth and development by the insulin-like growth factors (IGFs) is well accepted and interventions that block the IGF axis as such are currently in development for cancer therapy (Jones *et al*., 2005). In addition, various components of this axis are modulated by dietary and pharmacological cancer interventions (Voskuil *et al*., 2005).

Insulin-like growth factor-binding protein-3 (IGFBP-3), one of six members of the IGFBP family that noncovalently bind to IGFs with high affinity, is the most abundant in human serum (for a review see Firth and Baxter, 2002). IGFBP-3 is a multifunctional protein that transports and stabilizes IGFs in circulation; modulates IGF bioactivity; inhibits the growth of cancer cells; and induces apoptosis of cancer cells. The effects of IGFBP-3 on cell growth and apoptosis involve both sequestering IGFs from their receptors and IGF-independent mechanisms that include: binding to retinoid X receptor (RXR) and modulation of nuclear signalling followed by nucleomitochondrial translocation of RXR/Nur77 and induction of rapid apoptosis (Lee *et al*., 2005); binding to membrane receptors (Huang *et al*., 2003); and antagonism of the recently described survival factor, humanin (Ikonen *et al*., 2003). We have recently reported the initial description of successful therapeutic use of IGFBP-3 as a cancer therapy *in vivo*, and demonstrated that combination treatment of IGFBP-3 and RXR ligand had a synergistic effect on apoptosis induction leading to substantial inhibition of prostate cancer xenograft growth (Liu *et al*., 2005).

We hypothesized that apart from apoptosis induction, IGFBP-3 might have direct effects on angiogenesis because: (1) IGFBP-3 contains a highly basic heparin-binding area, and specifically binds to vascular endothelial cell monolayers (Booth *et al*., 1996) in a manner that may affect vascular angiogenesis; (2) IGFBP-3 inhibits vascular endothelial growth factor (VEGF)-mediated survival of human umbilical vein endothelial cells (HUVEC) in an IGF-independent mechanism (Zadeh and Binoux, 1997; Franklin *et al*., 2003) and

may also affect angiogenesis *in vivo*; (3) IGFBP-3 is transcriptionally upregulated during hypoxia, a potent stimulator of angiogenesis (Diaz-Gonzalez *et al*., 2005) in endothelial cells (Koong *et al*., 2000); and (4) IGFBP-3 mRNA is predominantly expressed in the vascular endothelial cells of human (Fraser *et al*., 2000), rat (Erickson *et al*., 1993), and bovine corpus lutea (Brown and Braden, 2001), which suggest a possible involvement in angiogenesis regulation, perhaps as part of a feedback mechanism. Other reports also indicate that IGFBP-3 mRNA is more abundantly expressed in hypoxia-associated inflammatory angiogenesis (Tucci *et al*., 1998; Lee *et al*., 1999) and tumor endothelial cells (Schmid *et al*., 2003). Importantly, a recent publication identifies IGFBP-3 as a farnesyl transferase inhibitor-induced negative regulator of angiogenesis in head and neck squamous cell carcinoma (Oh *et al*., 2006).

Here, we report that IGFBP-3 has direct, IGF-independent inhibitory effects on angiogenesis. Solid tumors require a supply of blood vessels to survive, grow and metastasize (Folkman, 2004) and treatments that address these issues can be more effective than nonspecific chemotherapies. Our results reveal a unique mechanism by which IGFBP-3 exerts its tumor suppressive effects and supports further investigation into the clinical translation of IGFBP-3 as a neoadjuvant in prostate cancer therapy.

Results

IGFBP-3 inhibits the growth of 22RV-1 prostate cancer xenografts in vivo

To examine the effects of IGFBP-3 as a single therapy on inhibiting prostate cancer tumor cell growth *in vivo*, male severe-combined immunodeficiency (SCID) mice with 22RV-1 prostate cancer xenografts were given daily injections of saline, or IGFBP-3 (30 mg/kg/day intraperitoneally (i.p.)) for 16 days. Treatment with IGFBP-3 resulted in significant tumor size inhibition (40% growth inhibition, $P<0.005$; $n=10$) relative to control animals (Figure 1a). A greater effect (50% inhibition) was seen for IGFBP-3 therapy on tumor weight (Figure 1b). These studies show that treatment of IGFBP-3 as a single therapy inhibits the growth of 22RV1 prostate cancer xenografts.

Induction of tumor apoptosis by IGFBP-3

Inhibition of xenograft growth by IGFBP-3 and RXR ligand is associated with an increase in apoptosis and activated Caspase-3 (Liu *et al*., 2005). The effect of IGFBP-3 single therapy on apoptosis in this xenograft model was examined by light microscopic terminal nucleotidyl transferase-mediated nick end labeling (TUNEL) assay. Representative photographs are shown in Figure 2a. As shown in Figure 2b, quantification of TUNEL-positive cells was increased sevenfold in the IGFBP-3 treatment group over saline-treated tumors. We next assessed whether this effect of IGFBP-3 is associated with an *in vivo* activation of Caspase-3. Microscopic examination of tumor sections stained for Caspase-3 clearly showed an increased detection of Caspase-3 in the IGFBP-3 therapy group as compared with the control group (Figure 2c). Quantification of Caspase-3-positive staining was increased sevenfold in IGFBP-3 treatment group over saline-treated tumors (Figure 2d). To further evaluate if regulation of cellular proliferation is also involved in the actions of IGFBP-3 on prostate cancer xenografts, we stained the tumors with the proliferation marker proliferating cell nuclear antigen (PCNA), and as shown in Figure 2e, observed no difference between IGFBP-3 and saline treatment (quantified in Figure 2f), suggesting that IGFBP-3-mediated inhibition of tumor growth does not involve regulation of cell proliferation. Negative controls, in which PCNA antibodies were omitted, did not show any positive staining (data not shown). This is in agreement with our previous observation utilizing a lower dose of IGFBP-3 (Liu *et al*., 2005).

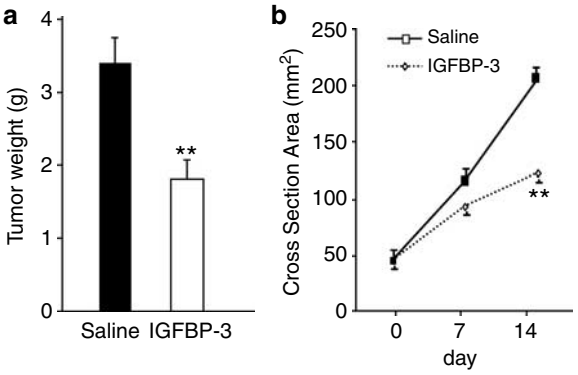

Figure 1 IGFBP-3 inhibits the growth of 22RV1 prostate cancer xenografts *in vivo*. (**a**) Tumor weights of 22RV1 CaP xenografts ($n=10$ per group) treated with or without IGFBP-3 (30 mg/kg/day), for 16 days. $^{**}P<0.005$ as compared with saline treatment. (**b**) Calculated tumor volume of 22RV1 CaP xenografts in this same experiment.

IGFBP-3 decreases vessel formation in vivo

Microvessel density, a measurement used for quantifying intratumoral angiogenesis activity, has been suggested as a valuable prognostic marker in prostate carcinoma (Weidner *et al*., 1993). To investigate if IGFBP-3 had any effect on intratumoral angiogenesis, we carried out immunohistochemical staining using an antibody against CD31, an endothelial cell-specific antigen, to evaluate the antiangiogenic effect of IGFBP-3 on 22RV1 tumor xenografts. As shown in Figure 3, a twofold increased number of CD31-positive microvessels and endothelial cells were found in the control group compared to the IGFBP-3-treated group. Our results indicate that IGFBP-3 is able to suppress prostate cancer angiogenesis through inhibiting microvessel formation.

IGFBP-3 inhibits both IGF- and VEGF-induced vascular formation

VEGF is one of the most potent angiogenic factors affecting endothelial proliferation, motility and vascular

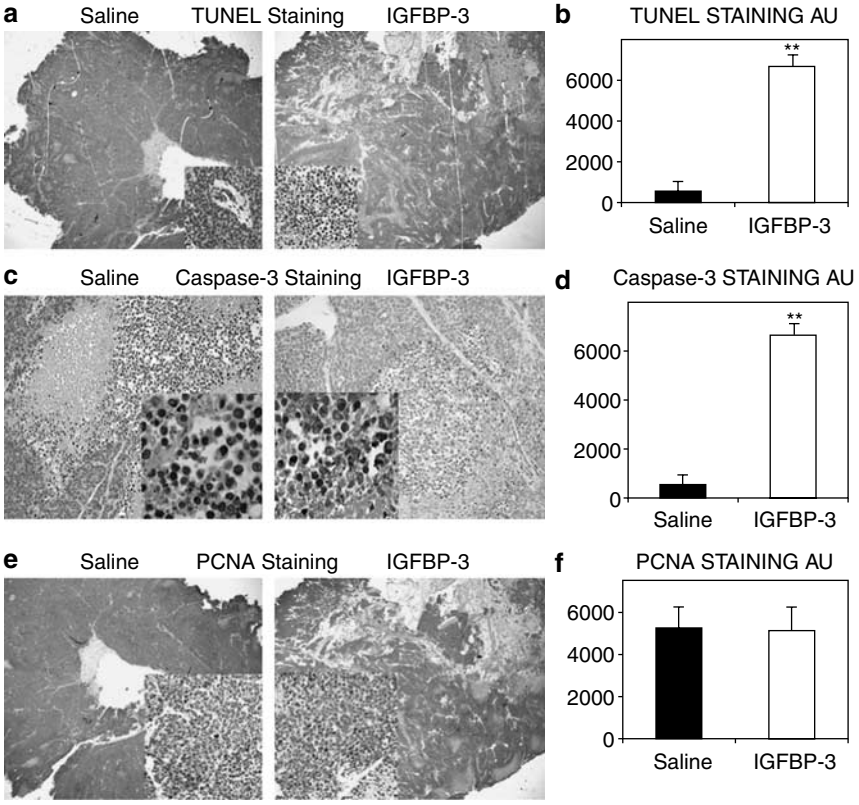

Figure 2 Inhibition of xenograft growth by IGFBP-3 is associated with an increase in apoptosis but does not involve a change in cell proliferation. (**a**) TUNEL immunohistochemistry of xenografts in control with saline treatment (left) and treatment with IGFBP-3 (right). (**b**) TUNEL pixel histogram quantitation. $^{**}P<0.005$ as compared with control ($n=10$ per group). (**c**) Microscopic examination of tumor sections stained for activated Caspase-3 antigen in control with saline treatment (left), and treatment with IGFBP-3 (right). (**d**) Quantification of Caspase-3-positive staining per pixel histogram ($n=10$ per group). (**e**) Proliferating cell nuclear antigen immunohistochemistry of xenografts in control with saline treatment (left), and treatment with IGFBP-3 (right). (**f**) Proliferating cell nuclear antigen pixel histogram quantitation ($n=10$ per group). $^{**}P<0.005$ as compared with saline control.

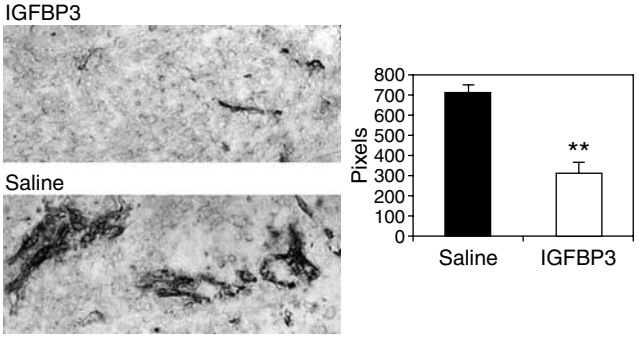

Figure 3 IGFBP-3 decreases vessel formation *in vivo*. Immunohistochemical staining of xenografts using CD31 antibody. (**a**) Control with saline treatment and IGFBP-3 treatment. (**b**) Quantification of CD31-positive staining by pixel histogram ($n=10$ per group).

permeability. VEGF binds with high affinity to the tyrosine kinase receptors *Flt-1* (VEGFR-1) and *Flk-1/KDR* (VEGFR-2) expressed by endothelial cells (Ferrara, 2001). VEGF expression by prostate cancer specimens is far greater than that by stromal cells of the normal prostate. These observations suggest that VEGF plays a role on tumor cell activation (autocrine regulation), in addition to paracrine actions, whereby it regulates endothelial cell (EC) functions and subsequent neovascular development (Jackson *et al*., 1997). We further investigated the effect of IGFBP-3 on VEGF-regulated vascular formation, using an established *in vitro* model of human endothelial cellular vessel formation in matrigel (Iwatsuki *et al*., 2005). IGFBP-3 alone has no effect on vascular formation in this assay; however, VEGF stimulated substantial new vascular complex formation. Importantly, VEGF-induced vessel formation was completely inhibited by IGFBP-3 cotreatment (Figure 4).

To further explore if effect of IGFBP-3 on VEGF-regulated vascular formation and to examine its effects on IGF-regulated vascular formation and to determine if this is an IGF-independent action, human endothelial cells on matrigel were treated individually with IGF-1, IGFBP-3, Long R3-IGF-1 (an IGF-1 analog which does not bind IGFBP-3) or their combination (Figure 4). IGF-1 stimulated vascular formation consistent with its described angiogenic effect (Hanahan and Folkman, 1996). IGFBP-3 blocked the angiogenic effect of IGF-1. Long R3-IGF-1, which does not bind IGFBP-3 also stimulates vascular formation and is partially blocked by IGFBP-3, indicating that IGFBP-3 interferes with

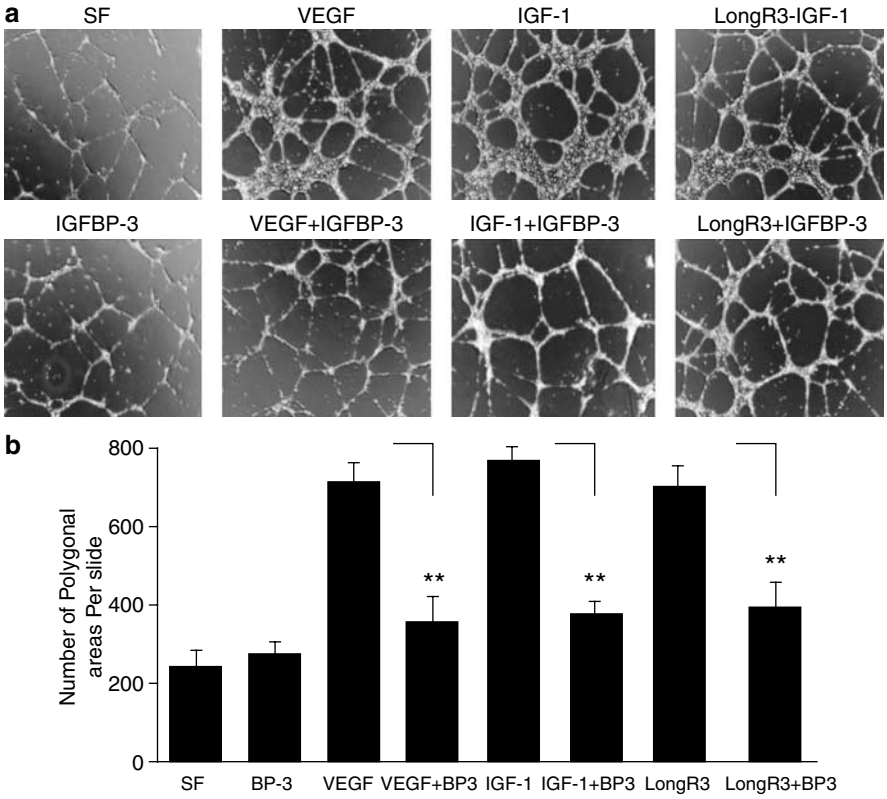

Figure 4 IGFBP-3 inhibits VEGF- and IGF-regulated vascular formation. (**a**) Representative pictures of human umbilical vein endothelial cellular vessel formation in matrigel under several treatment conditions. (**b**) Quantification of vessel formation ($n = 6$ per group).

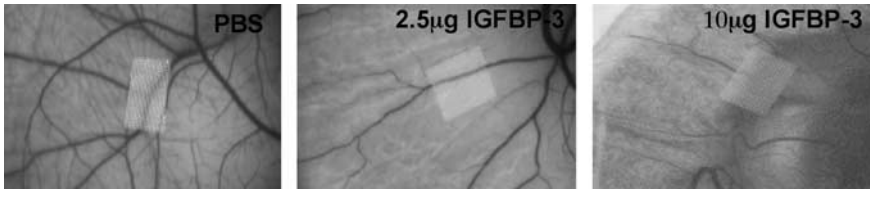

Figure 5 Antiangiogenic effects of rhIGFBP-3 on the CAM assay. Methylcellulose disks containing PBS, 2.5 and 10 μg of IGFBP-3 were implanted on CAMs of 6-day-old chick embryos. After 3–4 days, the formation of avascular zone was examined under a stereoscope. Each treatment was repeated in six chick CAMs with similar results.

IGF-induced vascular formation in part via an IGF-1-independent mechanism.

IGFBP-3 inhibits in vivo *angiogenesis*

To investigate the antiangiogenic activity in an *in vivo* setting, rhIGFBP-3 was tested on chicken embryo chick allantoic membrane (CAM) and murine matrigel plug angiogenesis assays. Compared to phosphate-buffered saline (PBS) control, IGFBP-3 at doses of 2.5 and 10 μg/disk completely inhibited the growth of new vascular vessels in all six tested chick embryos, which was measured by the formation of avascular zones (Figure 5). No inflammation was observed in these studies.

Basic fibroblast growth factor (bFGF)-induced angiogenesis has been considered as a model of tumor-derived neovacularization (Klauber *et al*., 1997). Figure 6 shows that the bFGF plugs were bright red and contained a large numbers of micro-blood vessels, which penetrated into the solidified martrigel and spread widely. There were no blood vessels in the PBS plugs (data not shown). However, the numbers of micro-blood vessels decreased in the plugs with both bFGF and different doses of rhIGFBP-3 in a dose-dependent manner. Quantification by image analysis showed that bFGF-induced neovascularizations were inhibited by 17 and 75% at the doses of 2.5 and 5 μg/ml rhIGFBP-3, respectively. Addition of 10 μg/ml of IGFBP-3 to FGF-2 was not significantly different from the 5 μg/ml dose.

Overexpression/ectopic expression of human IGFBP-3 inhibits vascular patterning in Zebrafish embryos in an IGF-independent manner

To assess the role of IGFBP-3 in blood vessel patterning *in vivo*, we overexpressed/ectopic expressed human

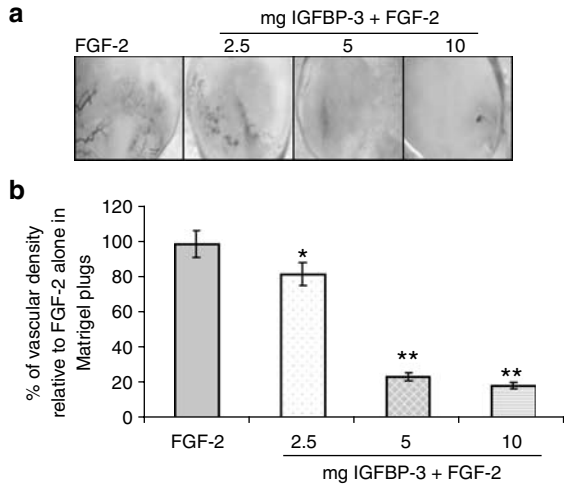

Figure 6 Antiangiogenic effects of rhIGFBP-3 on a bFGF-enriched matrigel plug. Mice received subcutaneous injection of 300 ml of a matrigel mixture containing FGF-2 with or without rhIGFBP-3. The animals were killed and dissected 2 weeks later, and the matrigel plugs were exposed and photographed. (**a**) A photograph of a representative matrigel plug from each group was shown. (**b**) Pixel histogram quantitation of percentages of vascular density in matrigels with FGF-2 and different doses of IGFBP-3 compared to FGF-2 alone was shown. Each group contained six animals. $*P<0.05$, $**P<0.01$ (FGF2 versus FGF2 + IGFBP-3, Student's *t*-test).

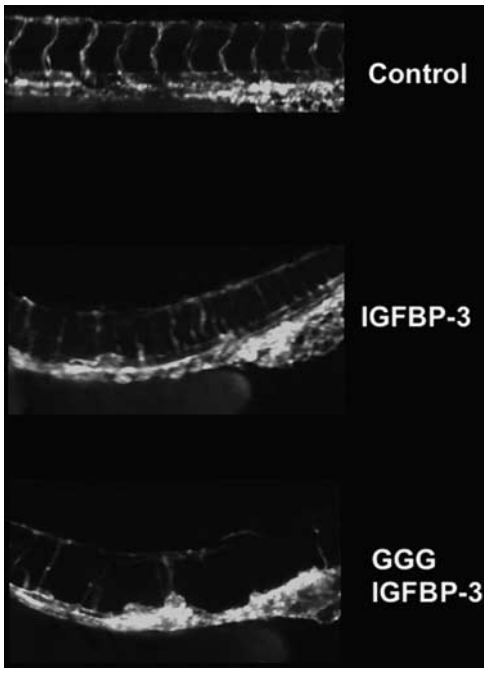

Figure 7 Overexpression/ectopic expression of human IGFBP-3 affects vascular patterning in Zebrafish embryos in an IGF-independent manner. Embryos from *flk1*:GFP transgenic Zebrafish were injected with 300 pg of capped mRNA derived by *in vitro* transcription from either wild-type or IGF-binding defective human IGFBP3 cDNA at one-cell stage and observed at 36 h.p.f. The fluorescent micrographs showing vascular patterning reflected by GFP expression in control embryos are shown as are those from embryos injected with wild-type IGFBP-3 mRNA or mutant IGFBP-3 mRNA.

IGFBP-3 via injection of the corresponding mRNA into the cytoplasm of one-cell stage flk1:green fluorescent protein (GFP) transgenic Zebrafish embryos. flk1:GFP represents a stable integration of a GFP reporter gene driven by the promoter of VEGF receptor-2 (flk1) and it utilized in the rapid, high-throughput screening of antiangiogenesis drug screening (Cross *et al*., 2003). As the vascular endothelial cells are labeled with green fluorescence, the blood vessels can be easily visualized in live embryos in this transgenic fish line. As shown in Figure 7, introduction of IGFBP-3 (300 pg of mRNA) resulted in defects of vascular patterning in the trunk and tail regions (Figure 7) in 36 h.p.f. (hours post fertilization) embryos. Intersegmental vasculogenesis is severely affected. Vessels are lacking and the remaining vessels are abnormally positioned. In addition, regional somites are compressed compared to control fish. To test whether the observed effect is dependent on the function of IGFBP-3 in IGF-sequestration, mRNA from a mutant form of IGFBP-3 which is defective in IGF binding was injected into flk1:GFP embryos in parallel with wild-type form. The phenotype in embryos injected with the mutant form is essentially indistinguishable from that with wild-type IGFBP-3, indicating that the effect of IGFBP-3 on vasculogenesis is independent of IGF sequestration. These apparently dorsalized embryos resemble previously characterized Zebrafish mutants such as the notochord mutant ntl, which shows defects in notochord differentiation (Odenthal *et al*., 1996) as well as the dominant-negative IGF-1R overexpression mutant (Eivers *et al*., 2004).

Discussion

In prostate cancer, as in other cancers, tumor-associated angiogenesis is a crucial step in the process of tumor growth, invasion and metastasis (van Moorselaar and Voest, 2002). Previous studies on prostate cancers have demonstrated a correlation between microvessel density, pathological stage and Gleason score (Arakawa *et al*., 1997; Bettencourt *et al*., 1998; Bono *et al*., 2002). Therefore, inhibiting vessel formation offers hope to reduce the morbidity and mortality from prostate cancer, and solid tumor cancers in general (Folkman, 1971).

Two reports have shown that IGFBP-3 inhibited the growth of bovine aortic endothelial cells (Delafontaine *et al*., 1996) and the IGF-/VEGF-induced proliferation of HUVEC (Franklin *et al*., 2003). In addition, IGFBP-3 is inhibitory for both proliferation and *in vitro* angiogenesis of the mouse endothelial progenitor cell line AEL-R1/LRT-*Runx1* in a manner independent from growth inhibitory effects of IGFBP-3 (Iwatsuki *et al*., 2005). This was shown to be specific to IGFBP-3 as IGFBP-6 was not inhibitory for *in vitro* angiogenesis of AEL-R1/LRT-*Runx1* cells, even at a higher concentration.

It has been reported that VEGF and transforming growth factor 1 downregulate the expression

of *IGFBP-3* in aortic endothelial cells (Erondu *et al*., 1996; Dahlfors and Arnqvist, 2000), whereas IGF, tumor necrosis factor-α and interleukin-1 upregulate it in endothelial cells or articular chondrocytes (Olney *et al*., 1995; Erondu *et al*., 1996). Thus, *IGFBP-3* may be a downstream effector for many growth regulatory cytokines and its transcription must be regulated differently depending on the cell type. Indeed, knockdown of IGFBP-3 expression by RNA interference or neutralizing antibodies blocked the antiangiogenic effect of the farnesyl transferase inhibitor SCH66336 in head and neck squamous cell carcinoma (Oh *et al*., 2006).

IGFBP-3 is known to be overexpressed during the angiogenic phase of the corpus luteum in rats, primates and humans (Erickson *et al*., 1993; Fraser *et al*., 1998, 2000). Other reports also indicate that IGFBP-3 mRNA is more abundantly expressed in hypoxia-associated inflammatory angiogenesis (Tucci *et al*., 1998; Lee *et al*., 1999) and tumor endothelial cells (Schmid *et al*., 2003). Indeed, hypoxia is the major pathophysiological condition regulating angiogenesis, and increased angiogenesis in response to hypoxia is part of an adaptive response aimed at achieving increased delivery of oxygen and nutrients to tissues (Acker and Plate, 2003). IGFBP-3 is induced by hypoxia (Grimberg *et al*., 2005) and may be involved in the normalization of tumor vasculature, modulating the abnormal structure and function of tumor vasculature (Jain, 2005).

Chan *et al*. (1998) demonstrated that plasma level of IGFBP-3 was a predictor of advanced-stage prostate cancer (relative risk = 0.2, 95% confidence interval 0.1–0.6 for the highest versus the lowest quartiles of IGFBP-3). Lee *et al*. (2002) has reported that the overexpression of rhIGFBP-3 by an adenoviral vector, Ad5CMV, inhibited the growth of non-small-cell lung cancer cells in tumor xenografts. Singh *et al*. (2004b) showed that the antitumor effects of both grape seed extract and inositol hexaphosphate (Singh *et al*., 2004a) were associated with a concomitant rise in serum IGFBP-3 and importantly, suppression of angiogenesis as measured by tumor vessel immunohistochemistry and serum VEGF levels. Silibinin, an antioxidant flavonoid, also inhibits prostate cancer xenograft growth and was associated with a decrease in tumor VEGF staining as well as increased intracellular IGFBP-3 staining (Singh *et al*., 2003) in mice.

Evidence for IGF-independent actions of IGFBP-3 include: (1) effects on cells that lack a functional type 1 IGF receptor (Valentinis *et al*., 1995); (2) IGFBP-3 binds other protein partners (receptors) that are not associated with IGFs (Liu *et al*., 2000; Huang *et al*., 2003; Ikonen *et al*., 2003); and as supported in the current study, (3) IGF analogs that do not bind IGFBP-3 fail to block IGFBP action (Franklin *et al*., 2003); and (4) IGFBP-3 mutants that do not bind IGFs maintain biologic actions (Chan *et al*., 2005). In the present study, we further support this concept by demonstrating antagonism of VEGF action and reporting data utilizing a mutant form of IGFBP-3 with greatly reduced IGF binding in Zebrafish.

In summary, these data provide evidence for the first time that rhIGFBP-3 has direct inhibitory effects on angiogenesis. As invasion and angiogenesis are important determinants of tumor progression, this newly described function of IGFBP-3 could have important relevance to both the prediction of cancer progression as a biomarker and cancer therapy as a therapeutic target. Collectively, these results suggest that inhibition of human prostate cancer growth by IGFBP-3 is associated with its *in vivo* antiproliferative, proapoptotic and antiangiogenic efficacy and supports further research into the potential clinical use of IGFBP-3 or pharmacological inducers of IGFBP-3 as neoadjuvant approaches for patients with prostate cancer.

Materials and methods

Cell culture

22RV1 cells were from ATCC (Manassas, VA, USA) and maintained as directed. HUVEC and Microvascular Endothelial Cell Growth Medium Bullet Kit-2 (EGM-2-MV Bullet Kit) were purchased from Clonetics (San Diego, CA, USA) and maintained as directed. All cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

Tumor xenografts

22RV1 xenograft tumors were generated by injection of 1×10^6 cells in 200 μl mixed at a 1:1 dilution with matrigel in the right flank of male SCID mice. Tumors were established for 2 weeks before the start of treatment. Ten SCID mice with 22RV1 tumors were treated daily with saline or IGFBP-3 (30 mg/kg/day), given by daily i.p. injections for 16 days. The length and width of the mass located at the site of injection of the 22RV1 cells were measured with calipers and recorded once a week. The mice were killed at day 16. Tumors were harvested, weighed, fixed in formaldehyde and embedded in paraffin. Animal care was in accordance with current regulations and standards of the National Institutes of Health, as well as our institutional guidelines for animal care. All animal experiments were approved by the animal research committee of the institutional review board.

Tumor immunohistochemistry

Paraffin-embedded sections were prepared from 22RV1 tumors harvested on day 16. After deparaffinization of tissue section, apoptotic DNA fragments were labeled by terminal deoxynucleotidyl transferase, and detected by antidigoxigenin antibody conjugated to fluorescein (ApopTag fluorescein *in situ* apoptosis detection kit, Chemicon, Temecula, CA, USA). Cells were examined at $\times 40$ using an inverted fluorescence microscope (Axiovert 135M, Carl Zeiss, New York, NY, USA). Apoptotic staining was quantified by pixel histogram (Adobe Systems, Mountain View, CA, USA) and confirmed by manual counting ($r = 0.98$) by counting the positive cells (brown-stained), as well as the total number of cells in 10 arbitrarily selected fields by an independent observer. Indirect immunohistochemistry was performed with Vectastain Elite ABC kit (Vector Labs, Burlingame, CA, USA) using 3,3′-diaminobenzidine as a chromogen and quantitated as per TUNEL assay above. Sections were incubated with mouse monoclonal antibodies against PCNA (Ab-1, 1:2500; Oncogene Science, Manhasset, NY, USA), CD31 (JC70A, 1:20; Dako Corp., Carpinteria, CA, USA),

activated Caspase-3 antibody (Sigma, St Louis, MO, USA) overnight at 4°C in a humidified chamber. Negative controls were treated with only Tris-buffered saline under the same conditions.

In vitro *vascular formation matrigel assays*
HUVEC cells (1.5×10^5) were resuspended in 1 ml of StemPro-34 SFM complete medium (Invitrogen, Carlsbad, CA, USA) with or without recombinant human IGFBP-3 or VEGF (Sigma, St Louis, MO, USA) and overlayed on a Biocoat matrigel basement membrane (BD Biosciences, San Jose, CA, USA) in a six-well plate. After a 12–14 h incubation at 37°C, the number of polygonal areas formed by vascular tube-like structures was counted under a microscope for each well (Iwatsuki *et al*., 2005).

Materials
Recombinant human IGFBP-3 and IGF-1 were obtained from INSMED Corp. (Glen Allen, VA, USA), aliquoted and stored at −80°C. The activity of rhIGFBP-3 for both IGF-I-binding and -inhibitory effect on cell proliferation of human breast cancer MCF7 cells was confirmed by Western ligand blotting and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described previously (Pratt and Pollak, 1994), respectively, before the experiments (data not shown). bFGF and the rabbit antiserum against human IGFBP-3 were purchased from Upstate Inc. (Lake Placid, NY, USA). Long R3-IGF-1 was obtained from Gropep (Adelaide, Australia). Matrigel (11.46 mg/ml) was from Becton Dickinson Labware (Bedford, MA, USA).

Chicken embryo chorioallantoic membrane assay
Three-day-old fertilized white Leghorn eggs were cracked, and chicken embryos with intact yolks were carefully placed in 20×100 mm plastic Petri dishes. After 6 days of incubation in 3% CO_2 at 37°C, a disk of methylcellulose containing 10, 5 and 2.5 μg of rhIGFBP-3 dried on a nylon mesh (3×3 mm) was implanted on the CAM of individual embryos. The nylon mesh disks were made by desiccation of 10 ml of 0.45% methylcellulose in water. After 3–4 days of incubation, embryos and CAMs were examined for the formation of new blood vessels in the field of the implanted disks by a dissecting microscope. Disks of methylcellulose containing PBS were used as negative controls (Cao *et al*., 1998).

Murine matrigel plug angiogenesis assay
Angiogenesis was assayed as the growth of blood vessels from subcutaneous tissue of mice into a solid gel of reconstituted basement membranes containing the test sample. Matrigel in liquid form at 4°C was mixed with bFGF (1 μg) and with PBS, or 2.5, or 5 or 10 μg/ml rhIGFBP-3, and then injected into the abdominal subcutaneous tissue of six mice/group. At body temperature, the matrigel rapidly solidifies. Mice were killed 2 weeks later, and the matrigel plugs were exposed for photography (Bagheri-Yarmand *et al*., 1999).

Zebrafish mRNA microinjection
Templates for transcription were prepared by linearizing plasmids pcDNA3IGFBP3 (wild-type IGFBP-3) and pcDNA1GGGBP-3 (non-IGF-binding GGG mutant of IGFBP-3 (Buckway *et al*., 2001)) with *Sma*I. Capped mRNA was synthesized *in vitro* using the mMESSAGE mMACHINE Kit (Ambion, Austin, TX, USA). After purification as recommended by the manufacturer, RNA was dissolved in nuclease-free water at a concentration of approximately 500 ng/μl. The RNA was diluted to a final concentration of 100 ng/μl and injected into the cytoplasm of one-cell stage *flk1*:GFP Zebrafish embryos.

Fluorescence microscopy
Embryos were examined under a fluorescein isothiocyanate filter on a Zess microscopy (Zeiss Axioplan-2, Thornwood, NY, USA). Pictures represent an area of 500×500 μm imaged with a $\times 20/0.7$ NA HC PlanApo lens. GFP was detected at a spectral range from 507 to 550 nm.

Statistical analysis
All *in vitro* experiments were repeated at least three times. Means ± s.d. are shown. Statistical analyses were performed using analysis of variance tests using InStat (GraphPad, San Diego, CA, USA). Differences were considered statistically significant when $^*P < 0.05$ and when $^{**}P < 0.005$.

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